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Relationship of Retrovirus Polyprotein Cleavages to Virion Maturation Studied with Temperature-Sensitive Murine Leukemia Virus Mutants

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Murine leukemia virus mutants *ts*3 (Moloney) and *ts*24 (Rauscher) both formed late-budding structures on the cell membrane at restrictive temperature. They both accumulated core polyproteins $Pr65^{gag}$ and $Pr180^{gag\text{-}pol}$ in cell membranes, but the envelope precursor was rapidly turned over. After shift to permissive temperature in the presence of cycloheximide, the accumulated precursors were sequentially cleaved via discrete intermediates both during the final stages of the budding process and in newly released virions to yield the finished virion core proteins and reverse transcriptase. The precursor form of reverse transcriptase was not enzymatically active and became activated partially or entirely inside released virions.

Three primary polypeptide translation products of the murine retrovirus genome have been defined. These are: (i) Pr^{env}, an 80,000- to 90,000-molecular-weight glycosylated precursor, translated on membrane-bound ribosomes (2, 6, 12, 17, 18, 34), that upon cleavage yields the virion envelope proteins gp70, p12E, and p15E; (ii) $Pr65^{gag}$, a 65,000-molecular-weight precursor, encoded on cytoplasmic ribosomes and possibly derived from a larger $Pr75^{gag}$, that is cleaved to form the major internal virion protein, p30, and three other low-molecular-weight proteins (4, 10, 27, 29, 30); and (iii) $Pr180^{gag\text{-}pol}$, a polyprotein of estimated molecular weight 180,000 made in small quantities that contains both $Pr65^{gag}$ and the virion reverse transcriptase, p85. $Pr180^{gag\text{-}pol}$ has been demonstrated both by in vitro translation using virion-derived 35S RNA as template and by in vivo labeling experiments (1, 11, 13, 19, 20, 23, 24, 30).

The precise mechanisms by which these proteins or their cleavage products aggregate at the cell membrane to form the viral budding structure are unknown. The interrelationship of macromolecular assembly and specific proteolytic cleavage is difficult to assess in cells infected by wild-type virus because all stages of assembly and processing are occurring simultaneously. In this report we describe the processing of murine leukemia virus (MuLV) polyproteins in cells infected by temperature-sensitive (*ts*) mutants Moloney *ts*3 (37) and Rauscher *ts*24 (28), both blocked at late stages of assembly. These mutants fail to cleave $Pr65^{gag}$ and $Pr180^{gag\text{-}pol}$ but not $Pr80\text{-}90^{env}$ at the restrictive temperature. The proteins all collect at the plasma membrane, and large numbers of late-stage budding structures are evident. Temperature shiftdown experiments in the presence of cycloheximide demonstrate that preformed $Pr65^{gag}$ and $Pr180^{gag\text{-}pol}$ can be cleaved. Cleavage of both precursors can be demonstrated in released virions and may also occur before release of buds from the cell. Enzymatically active reverse transcriptase is only demonstrable after cleavage. The genetic site of the primary defect in these mutants could not be ascertained from such experiments.

MATERIALS AND METHODS

Viruses and cells. MuLV Moloney strain *ts*3 (37) and Rauscher strain *ts*24 (28) were gifts from P. Wong, University of Illinois, and S. Aaronson, National Cancer Institute (NCI), respectively. Both mutants were cloned upon arrival in this laboratory in a previously cloned line of NIH/3T3 fibroblasts. The ratio of virion budding at 32.5/39°C was 100- to 500-fold for *ts*3 and 200- to 1,000-fold for *ts*24 assayed by reverse transcriptase release; similar results were previously found using other cell lines infected by these mutants (28, 35, 37). Cloned wild-type Moloney MuLV in NIH/3T3 fibroblasts has been previously described (7). Cell lines were maintained at 37°C for routine passage in Dulbecco-modified Eagle (DME) medium (Grand Island Biological Co.) with 10% calf serum.

Immune sera. Rabbit antisera to Moloney MuLV p30, gp70, and reverse transcriptase were prepared and characterized for specificity as previously described (24, 33). Goat antiserum to disrupted Moloney MuLV virions and purified p30 was received from R. Wilsnack, NCI. Antiserum from rats regressing a syn-

geneic lymphoma induced by Gross-type MuLV (anti-C58NTD) was a gift from I. Weissman, Stanford Medical School.

Metabolic labeling. Subconfluent cell monolayers (10-cm petri dishes, Falcon Plastics) were labeled with 2.5 ml of [^{35}S]methionine (New England Nuclear Corp., Boston, Mass.) at 50 μCi/ml in DME containing 1/50 the normal methionine concentration or ^{14}C-labeled mixed amino acids (10 μCi/ml) in DME with 1/100 the normal amino acid concentration, at the time and temperature indicated in the text. Cells were labeled with [^{32}P]orthophosphate at 50 μCi/ml in phosphate-free minimal essential medium as described in the text. In some experiments, cultures were shifted to permissive temperature (32.5°C) in complete DME containing 50 μg of cycloheximide per ml. This concentration of cycloheximide decreased protein synthesis to 2 to 5% that of control cells.

Immunoprecipitation. All procedures for extraction and processing of immunoprecipitates were carried out at 0 to 4°C. Cells were lysed, scraped into extraction buffer (1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 0.01 M NaH_2PO_4-NaH_2PO_4, 0.1 M NaCl, pH 7.5) at 1×10^6 to 2×10^6 cells/ml, and then clarified at 2,000 $\times$ *g* for 15 min. To preclear, extracts of 4 to 5 ml were incubated overnight with 20 μl of normal rabbit serum and 200 μl of 10% (vol/vol) Formalin-fixed *Staphylococcus aureus* Cowen strain I (14) and then reclarified at 150,000 $\times$ *g* for 2 h. This removed nonspecific adsorption to the immune adsorbent. Immune precipitates were made by incubating up to 1 ml of extract with 5 μl of immune serum overnight; 100 μl of a concentrated lysate (2×10^7 cells/ml) of uninfected NIH cells clarified at 150,000 $\times$ *g* for 2 h was included to compete out normal cell constituents that might precipitate. *S. aureus* (50 μl of 10% [vol/vol] stock) was then added for 2 h. The pellets were washed three times in lysis buffer and suspended for SDS-polyacrylamide gel electrophoresis in 1% SDS, 1% 2-mercaptoethanol, 50 mM Tris, pH 6.8, and 10% glycerol.

SDS-polyacrylamide gel electrophoresis. Linear 20 to 5% polyacrylamide gradient gels were prepared and used for electrophoresis as previously described (15, 34). Gels were fixed and processed for fluorography as previously described (5).

Reverse transcriptase. Reverse transcriptase was assayed with exogenous template and primer as previously described (22). Glycerol gradient centrifugation was as previously described (8).

Membrane fractionation. Total cell membranes were prepared by hypotonic swelling and Dounce homogenization as previously described (9, 34).

Electron microscopy. Cell monolayers were rapidly chilled to 0°C, washed extensively with phosphate-buffered saline (PBS), fixed in situ for 2 h at 0°C with 2% buffered glutaraldehyde, washed with PBS, and then processed and embedded with graded alcohol dehydration. Sections cut perpendicular to the plane of growth were post-stained with uranyl acetate and lead citrate and viewed with a Philips EM201 electron microscope.

Virion protein and precursor nomenclature. The convention of August et al. (3) with modifications of the NCI Tumor Viral Immunology Workshop (March 1977) for naming virion proteins, glycoproteins, phosphoproteins, and their precursors was followed. Molecular weights were estimated by comparison with standard protein markers (cytochrome *c*, immunoglobulin heavy and light chains, and bovine serum albumin) and known viral proteins of vesicular stomatitis virus and adenovirus 2.

RESULTS

Morphological block in assembly of *ts*3 and *ts*24. Cloned isolates of wild-type Moloney MuLV, *ts*3 (Moloney), and *ts*24 (Rauscher), all grown on the identical cell background, were examined by electron microscopy after growth at both permissive (32.5°C) and restrictive (39°C) temperatures. Cell monolayers were washed and then fixed in situ before dehydration. Sections were cut perpendicular to the plane of growth to allow for better orientation, as shown in a low-power view of a wild-type Moloney MuLV-infected cell (Fig. 1A). Wild-type virus-infected cells at either temperature or cells infected with *ts*3 or *ts*24 at 32.5°C contained budding structures in all stages of development, with an excess of apparently completed extracellular particles. Both *ts*3- and *ts*24-infected cells grown at 39°C accumulated large numbers of late-budding structures as shown in Fig. 1B and 1D (36, 38). Early-budding forms (Fig. 1C) were seen at a much lower frequency in cells infected by both mutants (Table 1).

Polyprotein precursors. The MuLV genome is translated into three major polyproteins that are later cleaved to form the virion proteins (4, 11, 18, 27). To analyze the size and immune reactivity of the wild-type Moloney MuLV viral proteins, infected and uninfected NIH cells were labeled for 20 min with [^{35}S]methionine, and the labeled proteins were immunoprecipitated with antisera prepared to purified p30, gp70, and p85 (reverse transcriptase) or to whole virus. The individual immunoprecipitates were then analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (Fig. 2). Uninfected cells contained virtually no immunoprecipitable radioactive bands (Fig. 2, control). From infected cells, anti-p85 (reverse transcriptase) precipitated Pr180$^{gag\text{-}pol}$ (lane 2); anti-p30 precipitated Pr65^{gag} and a lesser amount of Pr75^{gag} and Pr180$^{gag\text{-}pol}$ (lane 4); anti-gp70 precipitated Pr80^{env} (lane 3). The anti-whole-virus sera precipitated the three major precursors, and no other prominent bands were found (Fig. 2, lanes 5 and 6). During the 20-min labeling period, some *gag* cleavage products were produced including p30. No additional precursors were evident if ^{14}C-labeled mixed amino acids were used to label cells (not shown). Only Pr65^{gag} incorporated detectable ^{32}P after labeling of cells for

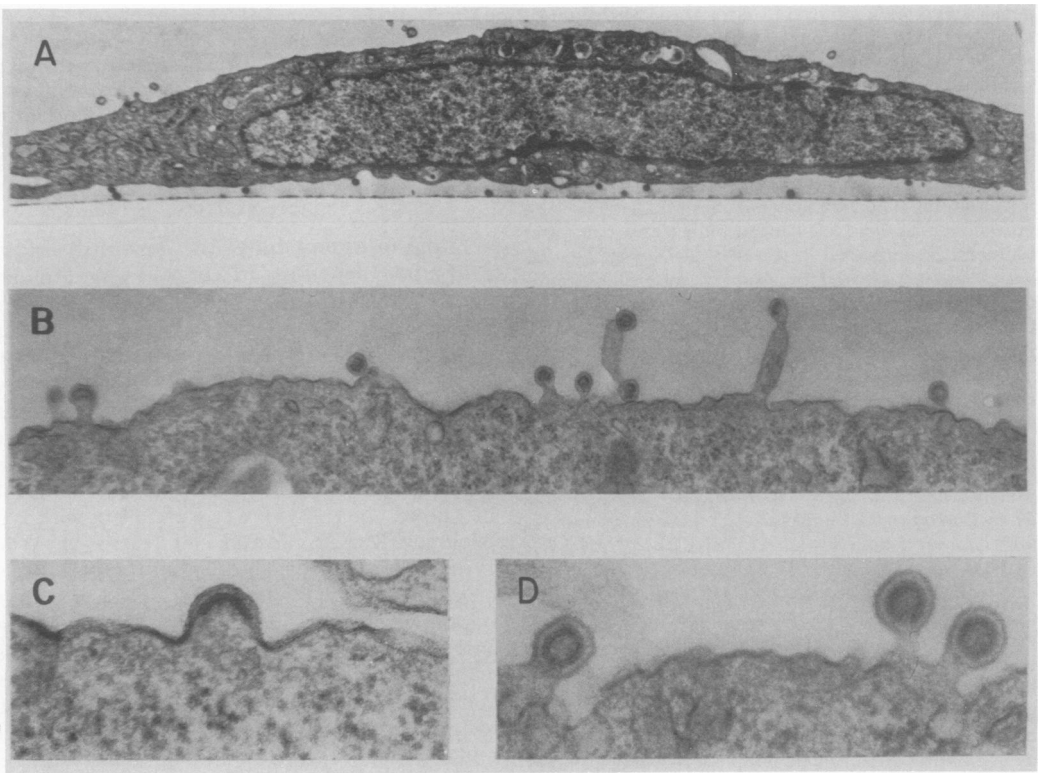

FIG. 1. *Electron microscopy of wild-type MuLV and temperature-sensitive mutant-infected cells. Cloned isolates of wild-type Moloney, ts3 Moloney, and ts24 Rauscher MuLV in infected NIH/3T3 cells were grown at 32.5°C and then shifted to 39°C with fresh medium for 6 h. Monolayers were then rapidly washed with ice-cold PBS, fixed in situ with glutaraldehyde-PBS at 0°C, and processed for electron microscopy. Sections cut perpendicular to the plane of the monolayer were used for better orientation. (A) Wild-type Moloney MuLV infected cell (×5,000). Darkly stained extracellular virions are found both above and below the cell. (B) ts24 Rauscher MuLV infected cell membrane (×20,000). Large numbers of late budding structures and fewer early budding forms and completed particles are seen. High-power (×45,000) view of an early budding (C) and late (D) form from ts3 Moloney-infected cells.*

20 min with $^{32}PO_4$ (Fig. 2).

Polypeptides of *ts* mutants. To analyze the polypeptides made by the *ts* mutants, NIH cells infected with either *ts*3 or *ts*24 were grown at 32.5°C, shifted to 39°C, and immediately labeled for 2 h with either [^{35}S]methionine or $^{32}PO_4$ (Fig. 3). During such a long labeling period, wild-type-infected cells showed extensive cleavage of the precursors (data not shown), but the mutant-infected cells showed mainly the three polyproteins Pr180$^{gag\text{-}pol}$, Pr65^{gag}, and Pr80^{env} or Pr90^{env}. (Note that Moloney MuLV makes a Pr80^{env} but Rauscher MuLV, wild type or mutant, makes a Pr90^{env}.) Immunoprecipitation of control, uninfected cells, in this experiment, showed a large number of immunoprecipitated bands with each of the antisera because the preclearing step used in other experiments was omitted for this experiment. A particular contaminant was the 190,000 to 200,000 band just above Pr180$^{gag\text{-}pol}$, which precipitated differently with each serum. This appears to be a normal cellular component, possibly the large external transformation-sensitive (LETS) protein. In spite of the background, each of the antisera precipitated the same polyprotein precursors as in the wild-type-infected, pulse-labeled cells of Fig. 2, but very little of the cleavage products from the precursors could be seen. If *ts*24-infected cells were labeled and then chased for 8 to 12 h, slow cleavage of Pr65^{gag} to a 50,000 to 55,000 form was observed. This polypeptide is presumably similar to that described by Reynolds and Stephenson (25) in *ts*24-infected cells.

After labeling for 2 h with $^{32}PO_4$ for *ts*3, only Pr65^{gag} showed detectable label. For the Rauscher *ts*24, both Pr65 and a slightly larger polypeptide (p70) were labeled. It is not clear whether this p70 represents a unique polypeptide or a heavily phosphorylated form of

$Pr65^{gag}$ (16, 26). Several other Rauscher stocks showed the same ^{32}P-labeling pattern (not shown).

We further investigated the properties of precursors in *ts* mutant-infected cells by cell fractionation studies. Membrane and cytosol fractions were isolated from *ts*24-infected cells labeled for 2 h at 39°C. Almost all of the $Pr65^{gag}$, $Pr180^{gag\text{-}pol}$, and $Pr90^{env}$ were associated with the membrane fraction (Fig. 4). Small amounts of Pr^{pol} and Pr^{gag} intermediates were seen, probably due to the occurrence of some processing during the multiple washing steps used to prepare the membrane preparation. Only after short pulse-labels (15 to 20 min) could appreciable amounts of $Pr65^{gag}$ and $Pr180^{gag\text{-}pol}$ be isolated in the cytosol fraction. $Pr90^{env}$ was found in the membrane fraction after the shortest labels as previously reported (34). Similar results have been obtained for *ts*3-infected cells (not shown).

TABLE 1. *Electron microscopy of wild-type and temperature-sensitive mutants of MuLV*[a]

Culture	Median and range of MuLV budding forms counted per cell section		
	Early buds	Late buds	Particles
Uninfected control	0 (0)	0 (0)	0 (0)
Wild-type Moloney	1 (0-3)	2 (1-6)	14 (5-23)
*ts*3 Moloney	2 (1-5)	20 (12-34)	3 (1-7)
*ts*24 Rauscher	3 (2-5)	22 (15-30)	3 (2-6)

[a] All cultures were grown at 32.5°C; then the medium was changed and the cultures were shifted to 39°C for 6 h before fixation and processing (see Materials and Methods). Sections from at least 10 cells were used for quantitation.

Pulse-chase analysis was performed to monitor the stability and rate of cleavage of these polyproteins at restrictive temperature. Preliminary experiments demonstrated that $Pr65^{gag}$ labeled during a 20-min pulse was stable for periods of up to 4 to 6 h for both *ts*3 and *ts*24. The $Pr80^{env}$ of *ts*3 and $Pr90^{env}$ of *ts*24 were found to turnover rapidly. A small fraction, though, was recovered as the defined cleavage products gp70 and P12E,15E. This rapid degradation of

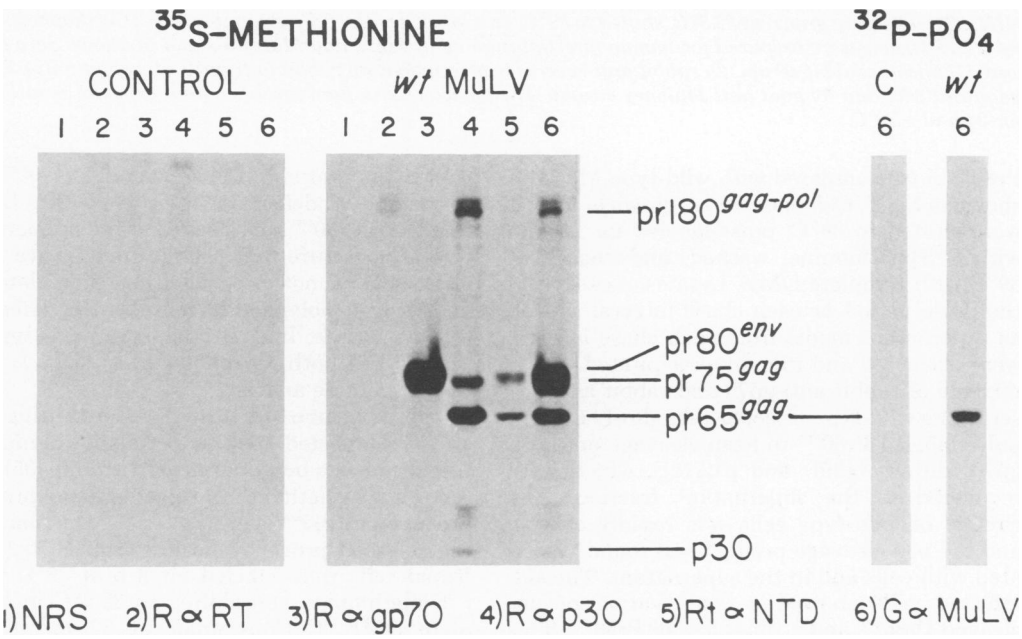

FIG. 2. *Pulse-labeled polyprotein precursors in cells infected by wild-type Moloney. Subconfluent monolayers of uninfected NIH/3T3 cells (control) and NIH/3T3 cells infected by a cloned wild-type Moloney MuLV were pulse-labeled (39°C, 20 min) with [^{35}S]methionine or [^{32}P]orthophosphate, lysed, and prepared for immunoprecipitation as described in Materials and Methods. The sera used were (1) normal rabbit serum, (2) rabbit anti-reverse transcriptase, (3) rabbit anti-gp70, (4) rabbit anti-p30, (5) rat anti-C58NTD, and (6) goat anti-Moloney MuLV virions. Immunoprecipitates were denatured in 1% SDS-1% 2-mercaptoethanol and electrophoresed on linear 20 to 5% gradient polyacrylamide SDS-gels (15, 34). The gels were fixed, processed for fluorography (5), and exposed for 24 h at −70°C to Kodak XR-5 film. Proteins and precursors are named according to the conventions of August et al. (3) and the NCI Tumor Viral Immunology Workshop (March 1977).*

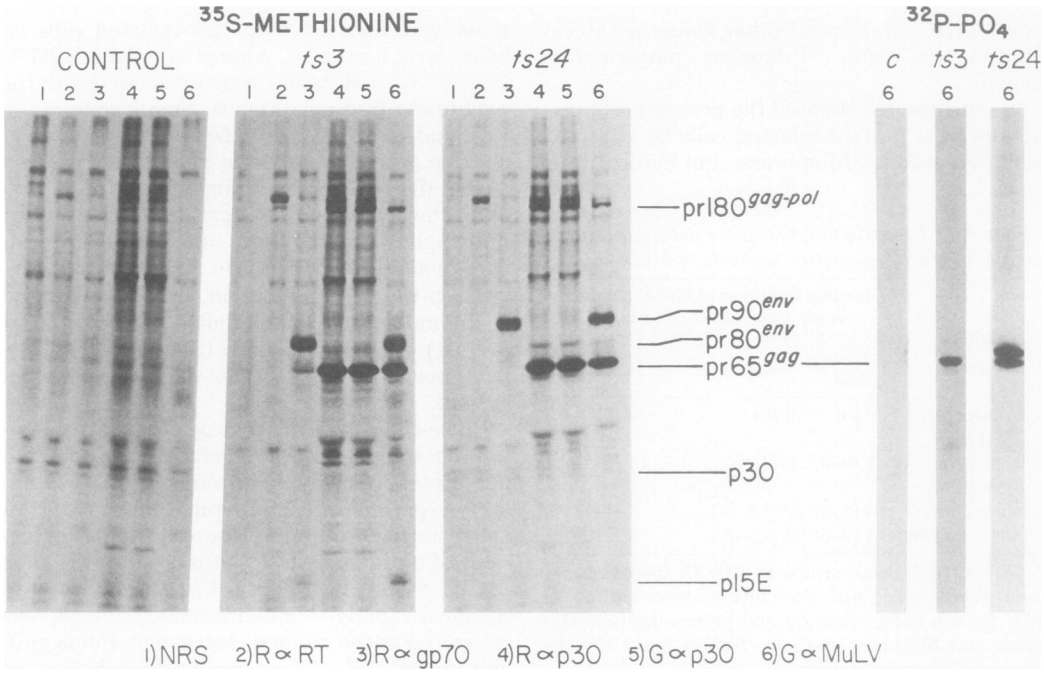

FIG. 3. *Accumulation of polyprotein precursors in cells infected by ts3 and ts24. Control or infected NIH/3T3 cells were grown at 32.5°C, shifted to 39°C, and labeled with [^{35}S]methionine or [^{32}P]orthophosphate for 2 h. Extracts were prepared for immunoprecipitation as in Fig. 2 and Materials and Methods. Sera used were (1) normal rabbit serum, (2) rabbit anti-reverse transcriptase, (3) rabbit anti-gp70, (4) rabbit anti-p30, (5) goat anti-p30, and (6) goat anti-Moloney virions. The gel was fixed, fluorographed, and exposed as in Fig. 2 for 24 h at −70°C.*

Pr80^{env} in *ts*3 compared with wild-type MuLV is shown in Fig. 5. Cell monolayers grown at 32.5°C were shifted to 39°C, pulse-labeled for 20 min with [^{35}S]methionine, washed, and chased at 39°C with complete DME. Lysates of cells from the pulse period or each chase interval as well as supernatant media from each chase interval were extracted and immunoprecipitated with a mixture of rabbit anti-gp70 and rabbit anti-p30 sera. The wild-type-infected cells slowly utilized pulse-labeled Pr80^{env} to form cleavage products gp70 (diffuse band) and p12,15E, which were recovered in the supernatant fraction. The Pr65^{gag} of wild-type cells was rapidly chased, and the p30 cleavage product was found associated with cells and in the supernatant. The *ts*3-infected cells showed a conservation of uncleaved Pr65^{gag} and a rapid loss of Pr80^{env}. This rapidly turning over Pr80^{env} was not quantitatively converted to gp70-p15E. In some experiments a small fraction (<10%) could be recovered as the appropriate cleavage products, but most Pr80^{env} from *ts*3 appeared to be degraded to unrecoverable products at restrictive temperature.

From the results, it appears that the two *ts* mutants both show accumulation of core precursor polyproteins (Pr65^{gag} and Pr180$^{gag\text{-}pol}$), suggesting a defect in their cleavage. Little Pr80^{env} or Pr90^{env} was cleaved at the nonpermissive temperature, but a large fraction was degraded. It is not possible from such data to define what polypeptide contains the defect in these mutants, but *ts*3 and *ts*24 have similar phenotypes both morphologically (Fig. 1) and by polypeptide analysis.

Temperature shiftdown. After shifting *ts*3- and *ts*24-infected cells to permissive temperature, there is a burst of released virions (35). To investigate whether the intracellular polyprotein precursors Pr65^{gag} and Pr180$^{gag\text{-}pol}$ contribute to the released progeny and are cleaved, *ts*24-infected cells were labeled for 2 h at 39°C with [^{35}S]methionine and shifted to 32.5°C in complete DME. Cycloheximide at 50 μg/ml was present after shiftdown to ensure that only premade polypeptides contributed to the yield. Both cell extracts and released particles were examined by specific immunoprecipitation and electrophoresis at various times after shiftdown.

Using anti-p85 (reverse transcriptase) serum, it was evident that in the cells and released virions, products other than Pr180$^{gag\text{-}pol}$ appeared after shiftdown (Fig. 6). In 20 min, two

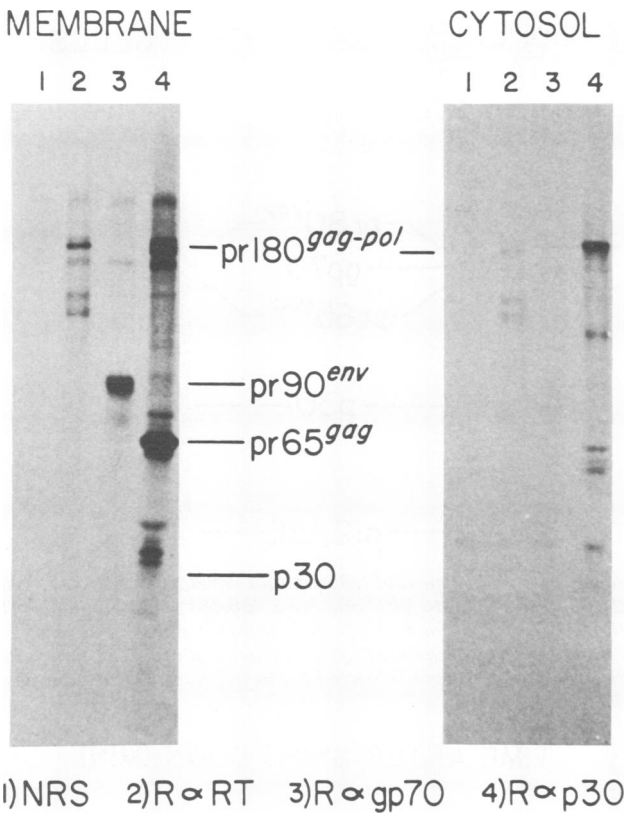

FIG. 4. *Binding of precursor polyproteins to cellular membranes. Cells infected by ts24 grown at 32.5°C were shifted to 39°C and labeled for 2 h with [^{35}S]methionine. Monolayers were rapidly chilled at 0°C, washed with iced saline, hypotonically swollen, and broken by homogenization with a Dounce homogenizer (9, 34). Nuclei and unbroken cells were removed at 1,000 × g for 10 min, and then membranes were pelleted at 40,000 × g for 25 min. Membranes and cytosol were prepared for extraction and immunoprecipitation. Sera used were (1) normal rabbit serum, (2) rabbit anti-reverse transcriptase, (3) rabbit anti-gp70, and (4) rabbit anti-p30. Samples were subjected to electrophoresis and fluorographed as in Fig. 2. The gel was exposed for 48 h.*

labeled polypeptides, Pr140-145^{pol} and Pr130^{pol}, became evident that were precipitated by anti-p85 but not by anti-p30. These presumably correspond to polypeptides called PrRT 1 and 2 by Jamjoom et al. (11). During longer chases of 40 and 60 min, mature p85 was recovered in the supernatant virions, but only trace amounts were associated with the cells. Some Pr180$^{gag\text{-}pol}$ was released before cleavage, as is more evident in Fig. 7. The high-molecular-weight band above Pr180$^{gag\text{-}pol}$ is a cellular protein (or proteins) that apparently binds to the immune complexes.

The anti-p30 serum detected in both cells and virions a series of intermediate cleavage forms after shiftdown along with uncleaved precursors. In virions, p30 built up with time, whereas Pr65^{gag} and the intermediate Pr40^{gag} remained evident throughout a 60-min chase period.

The anti-gp70 serum detected mainly Pr90^{env} in the cells over the 60 min after shiftdown. In the virions, however, only gp70 was recovered (p15E and p12E were not included in these gels).

In summary, the data of Fig. 6 show that after shiftdown, both Pr65^{gag} and Pr180$^{gag\text{-}pol}$ start to be cleaved and released into virions. Both uncleaved forms and intermediates were found in cells and in newly made particles. The final products, p30 and p85, build up with time in virions; p30 is maintained at a steady-state level in cells but p85 is not found in cells.

Some Pr65^{gag} and Pr180$^{gag\text{-}pol}$ were released from cells in uncleaved form after shiftdown. To determine whether these could be cleaved after release from cells, *ts*3 virions harvested at 20 min after shiftdown to 32.5°C were incubated at permissive temperature in serum-free medium (Fig. 7). Pr65, Pr180, and Pr130 were all reduced by this incubation, and appearance of finished reverse transcriptase was evident. Reduction of the *gag* intermediates and increase of p30 also occurred. Addition of nonionic detergent inhibited all of the in vitro conversions. These

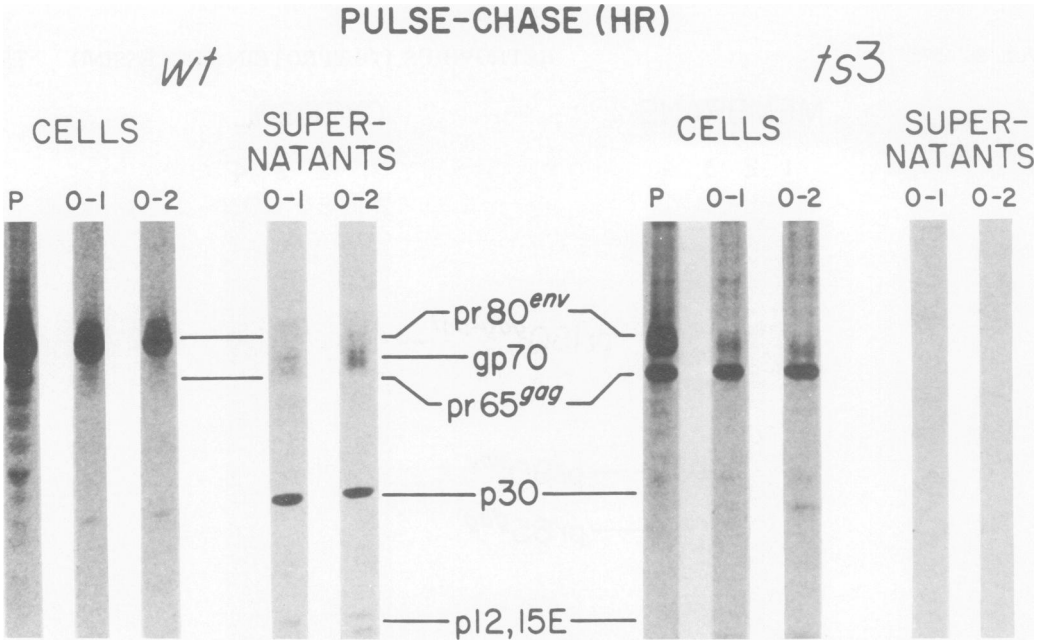

FIG. 5. *Pulse-chase analysis of wild-type and ts3 core and envelope precursors. Wild-type- or ts3-infected monolayers grown at 32.5°C were shifted to 39°C and pulse-labeled for 20 min with [^{35}S]methionine, washed, and chased at 39°C with complete DME for 1 or 2 h. Cells from the pulse period and each chase interval and the supernatant media from each chase interval were extracted and immunoprecipitated with a mixture of rabbit anti-p30 and anti-gp70. Samples were subjected to electrophoresis and fluorographed as in Fig. 2. The gel was exposed for 48 h.*

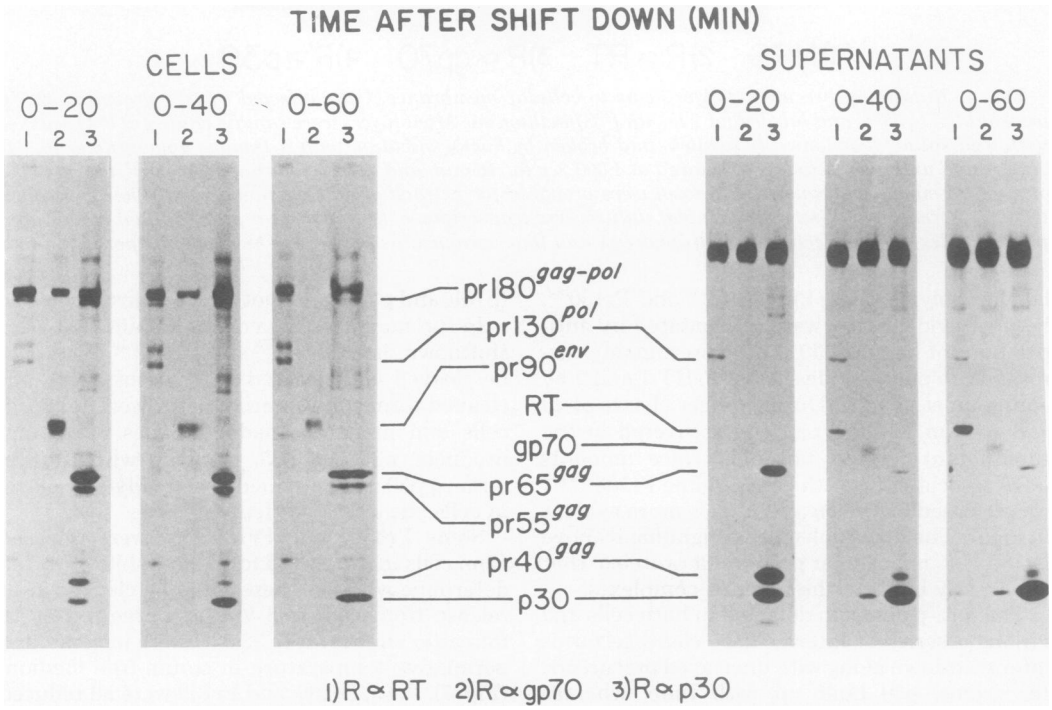

FIG. 6. *Cleavage of Pr65^{gag} and Pr180$^{gag\text{-}pol}$ during shiftdown of ts24-infected cells. Monolayers of ts24-infected cells grown at 32.5°C were shifted to 39°C, labeled for 2 h with [^{35}S]methionine, and then shifted down to 32.5°C in complete DME growth medium containing 50 μg of cycloheximide per ml. Both cells and supernatant fluids were harvested and extracted at various intervals and then processed for immunoprecipitation. Sera used were (1) rabbit anti-reverse transcriptase, (2) rabbit anti-gp70, and (3) rabbit anti-p30. Samples were subjected to electrophoresis and fluorographed as in Fig. 2. Cell extracts were exposed for 24 h, and supernatant fluid was exposed for 72 h at −70°C.*

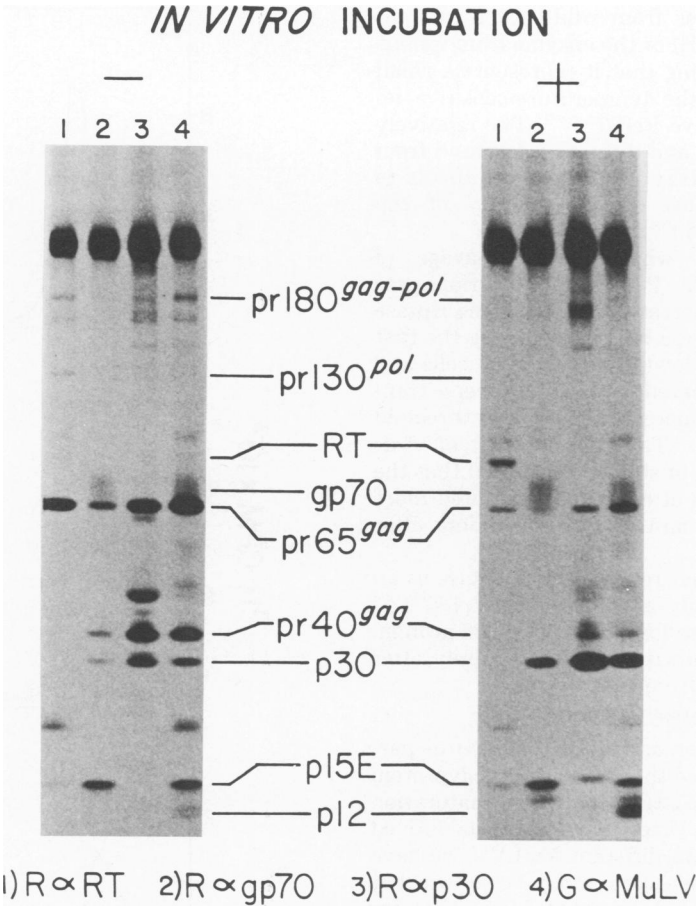

FIG. 7. *In vitro processing of Pr180$^{gag-pol}$ and Pr65^{gag}. Cells infected with ts3 were grown at 32.5°C, shifted to 39°C, labeled for 2 h with [^{35}S]methionine, and then shifted to 32.5°C in complete DME without serum containing 50 μg of cycloheximide per ml. Supernatant fluid collected from 0 to 20 min after shiftdown was rapidly chilled to 0°C and clarified at 1,000 × g for 10 min to remove any cells or large debris. One portion was further incubated at 32.5°C for 20 min, and then both were extracted for immunoprecipitation. Sera used were (1) rabbit anti-reverse transcriptase, (2) rabbit anti-gp70, (3) rabbit anti-p30, and (4) goat anti-Moloney virion. Samples were subjected to electrophoresis and fluorographed as in Fig. 2. The gel was exposed for 72 h.*

supernatant precursors were presumably packaged into virions because they were in a particulate fraction and had a density between 1.14 and 1.17 g/ml (unpublished observations).

Enzymatic acitvity of Pr180$^{gag-pol}$. Previous work has indicated that retrovirus-infected cells have little or no active intracellular reverse transcriptase (21). Cells infected by *ts*3 or *ts*24, however, build up Pr180$^{gag-pol}$, the precursor to reverse transcriptase at nonpermissive temperature. As shown above, the Pr180$^{gag-pol}$ is localized in cell membranes; therefore, to investigate whether Pr180$^{gag-pol}$ is enzymatically active, the reverse transcriptase activity of membranes of cells infected with wild-type MuLV, *ts*3, and *ts*24 was studied (Table 2). Cells were also shifted to permissive temperature in the presence of cycloheximide to study release of reverse transcriptase. Membranes of *ts* mutant-infected cells held at nonpermissive temperature had relatively little active enzyme. After shiftdown, enzyme activity in the membranes increased, and threefold more enzyme was released from the cells infected with *ts*3 and *ts*24 than from cells infected with wild-type virus (Table 2).

To investigate whether any of the enzyme activity found in membranes at nonpermissive temperature was Pr180$^{gag-pol}$, the size of the enzyme solubilized from membranes was investigated by glycerol gradient centrifugation (Fig. 8). The only defined peak of enzymatic activity was 85,000 in molecular weight (Fig. 8C), like

that of membranes from wild-type MuLV-infected cells (Fig. 8B) or the enzyme from virions (Fig. 8A), suggesting that it represents a small breakthrough of the temperature-sensitive lesions and not active $Pr180^{gag\text{-}pol}$. The relatively low incorporation and diffuse background from *ts*24 membranes (Fig. 8C) make it difficult to rule out some low specific activity of the $Pr180^{gag\text{-}pol}$ or $Pr130^{pol}$ precursors.

To investigate whether the cleavage of $Pr180^{gag\text{-}pol}$ and the Pr^{pol} forms in virions was paralleled by an increase of reverse transcriptase activity, virions were harvested during the first 15 min after shiftdown of *ts*3-infected cells and then further incubated for 30 min. Reverse transcriptase activity increased more than threefold during incubation (Table 3). Harvest of virus after 30 or 60 min of shiftdown showed that the threefold increase of enzyme represented more than another 15 min of collection time after shiftdown.

We conclude that reverse transcriptase as an active enzyme only appears when $Pr180^{gag\text{-}pol}$ has been cleaved to form the active p85 protein. This cleavage occurs mainly, if not entirely, after release of virions from cells.

DISCUSSION

Previous work on assembly of retrovirus particles had suggested that cleavage of polyprotein precursors was a late step in the maturation sequence (29, 38). Using two *ts* mutants derived independently from different MuLV's, we have confirmed this suggestion and have shown that the *ts* lesion(s) retards two of three polyproteins from cleaving. We have further been able to demonstrate that after shiftdown from restrictive temperature, preformed polypoteins are

TABLE 2. *Reverse transcriptase activity of cell membranes and virions from temperature-sensitive mutants*

Source	cpm × 10^{-3}/10^6 cells			
	Uninfected	Wild type	*ts*3	*ts*24
Cell membranes harvested from cells held at 39°C for 8 h	<1	25.0	1.6	3.8
Cell membranes harvested from cells shifted to 32.5°C for 1 h[a]	<1	20.5	5.7	24.3
Virions released from cells during 1-h shiftdown[a]	<1	34.0	110.0	87.5

[a] Cells held at 39°C were shifted to 32.5°C in fresh medium with 50 μg of cycloheximide per ml for 1 h, and then supernatant virions were pelleted. Total cell membranes pelleted after hypotonic swelling and Dounce homogenization (9, 34) were dissolved in 20 mM Tris, pH 8.3–1% Nonidet P-40. Reverse transcriptase was measured with an exogenous template·primer assay (22).

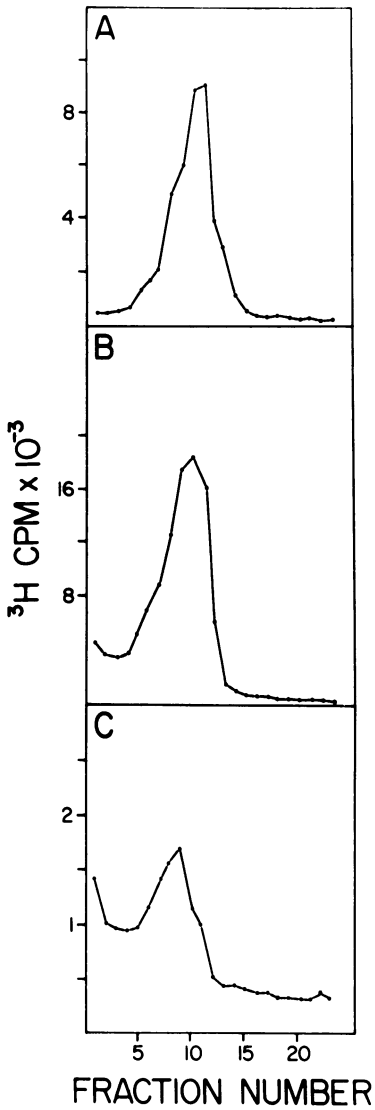

FIG. 8. *Glycerol gradient analysis of reverse transcriptase. Cellular membranes or virion samples of 100 μl were extracted in 1% Nonidet P-40 in 100 mM NaCl, 10 mM Tris, pH 7.5, 5 mM $MgCl_2$, 0.1 mM EDTA, and 10 mM dithiothreitol and layered on 5 ml of 40 to 20% glycerol gradients (SW50.1 rotor) containing the same buffer with 1% Nonidet P-40. Gradients were run at 45,000 rpm, 32 h, 4°C. Fractions were assayed for reverse transcriptase by using an exogenous template·primer assay (22). (A) Wild-type MuLV virions. (B) Cell membranes isolated by pelleting after hypotonic swelling and Dounce homogenization (9, 34) of 10^6 wild-type MuLV-infected cells grown at 39°C. (C) Cell membranes of 10^6 ts24-infected cells grown at 39°C.*

cleaved and that much of the cleavage takes place after release of virions from the cells.

Events of maturation. Although it is not evident from these data what is the precise genetic defect in *ts*3 and *ts*24, the mutants do reveal a crucial stage of the MuLV maturation sequence. Apparently, $Pr65^{gag}$ and $Pr180^{gag\text{-}pol}$, although made as soluble proteins, can migrate to the cell membrane and become organized in quite mature buds without proteolytic cleavage taking place. Presumably this is a normal stage in the MuLV budding process that has been "frozen" by the mutant function.

Both the Pr^{gag} and $Pr^{gag\text{-}pol}$ proteins are potential candidates for having the mutational lesion in these mutants. In principle, either could be prevented from a normal rate of cleavage by a mutation and thus block maturation.

The behavior of Pr^{env} in these mutants is less evident. It is much more unstable than the Pr^{env} of wild-type virus, and much of it degrades to unrecoverable products within an hour of its synthesis at nonpermissive temperature (Fig. 5). It is possible that the mutations are in the Pr^{env} gene and gave rise to the instability of the protein.

Because the precursor proteins labeled at nonpermissive temperature can be cleaved to their normal products after shiftdown, there is no irremedial defect in the MuLV proteins caused by the *ts* lesion. This fact has allowed us to demonstrate that Pr^{gag} cleavage can occur concomitant with the last budding stage and also in released virions. $Pr180^{gag\text{-}pol}$ can be cleaved to $Pr130^{pol}$ while buds are still cell associated, but cleavage to p85 occurs only after release of particles from the cell surface. We believe that these events occur during normal budding in the same order because Jamjoom et al. (11) showed that both $Pr65^{gag}$ and $Pr180^{gag\text{-}pol}$ cleavage are late events of the MuLV budding process. Opperman et al. (20) also showed for Rous sarcoma virus that $Pr180^{gag\text{-}pol}$ is cleaved in particles.

The fact that $Pr65^{gag}$ and $Pr180^{gag\text{-}pol}$ can be cleaved inside released virions incubated in the absence of serum or cells suggests that a protease is packaged in virions. Recent reports consistent with this idea have appeared (32, 39). Yoshinaka and Luftig (39) found that cleavage of Pr65 (they called it P70) required detergent treatment; we found that detergent inhibited cleavage. The times of incubation and concentrations were very different in the two experiments, and it seems likely that our conditions favor intravirion events, whereas their conditions allowed study of intervirion transfer of proteolytic factors.

Functional reverse transcriptase. It has previously been shown that infected cells make about 1 molecule of $Pr180^{gag\text{-}pol}$ for each 25 to 50 molecules of Pr^{gag} (11, 20). Both polypeptides appear to be initiated at the same place on a 35S mRNA. A readthrough from *gag* to *pol* is controlled by some mechanism that bypasses a UAG termination codon (24). The present studies indicate that $Pr180^{gag\text{-}pol}$ is enzymatically inactive. Because it is localized in membranes, it was possible to assay it independently of inhibitory factors that are present in the cytosol (21). Although $Pr180^{gag\text{-}pol}$ built up in membranes at restrictive temperature, very little reverse transcriptase was found in membranes compared to what could be released by shiftdown in the presence of cycloheximide (Table 2). Although a small amount of active 85,000-molecular-weight reverse transcriptase was found in membranes, no peak of active 180,000 or 130,000 form was found. Because appearance of active reverse transcriptase requires shiftdown and because reverse transcriptase activity increases after virions are released from cells (Table 3)—at the same time that p85 is cleaved from $Pr130^{pol}$—it appears that active reverse transcriptase is only made inside of released, newly made virions.

A mechanism that assures activation of functional reverse transcriptase in virions will prevent premature initiation of reverse transcription before infection. The ability to detect formation of the first internucleotide bond in activated virions (31) had previously suggested that functional enzyme might only appear after virion maturation is complete.

It seems likely that $Pr180^{gag\text{-}pol}$ associates with nascent buds through its $Pr65^{gag}$ portion; it would therefore aggregate into the nascent bud just like any Pr65 molecule, but would carry reverse transcriptase into the bud with the Pr65 moiety. The enzyme would then be cleaved off when the protease activity that cleaves Pr65 was activated. What Pr130 contains that is lacking in the p85 is unclear.

TABLE 3. *In vitro virion-bound generation of reverse transcriptase*

Time of collection after shiftdown (min)	cpm × 10^{-3} incorporated	+ 30 min cell-free incubation
0–15	14.2	47.5
0–30	32.2	—
0–60	80.3	—

[a] Cultures of *ts*3-infected NIH cells held at 39°C for 6 h were fed with serum-free DME containing 50 μg of cycloheximide per ml and shifted to 32.5°C for the indicated times. Supernatants were rapidly chilled to 0°C and clarified at 2,000 × *g* for 15 min, and then virus was pelleted at 100,000 × *g* for 60 min. One sample was reincubated for 30 min at 32.5°C before pelleting. Virus pellets were assayed for reverse transcriptase by an exogenous template·primer assay (22).

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